Exhibit H

Chronic Hypoxia Increases Endothelial Nitric Oxide Synthase Generation of Nitric Oxide by Increasing Heat Shock Protein 90 Association and Serine Phosphorylation

Yang Shi, John E. Baker, Chenyang Zhang, James S. Tweddell, Jidong Su, Kirkwood A. Pritchard, Jr

Abstract—Chronic hypoxia increases endothelial nitric oxide synthase (eNOS) production of nitric oxide (·NO) and cardioprotection in neonatal rabbit hearts. However, the mechanism by which this occurs remains unclear. Recent studies suggest that heat shock protein 90 (hsp90) alters eNOS function. In the present study, we examined the role of hsp90 in eNOS-dependent cardioprotection in neonatal rabbit hearts. Chronic hypoxia increased recovery of postischemic left ventricular developed pressure (LVDP). Geldanamycin (GA), which inhibits hsp90 and increases oxidative stress, decreased functional recovery in normoxic and hypoxic hearts. To determine if a loss in ·NO, afforded by GA, decreased recovery, GA-treated hearts were perfused with *S*-nitrosoglutathione (GSNO) as a source of ·NO. GSNO increased recovery of postischemic LVDP in GA-treated normoxic and hypoxic hearts to baseline levels. Although chronic hypoxia decreased phosphorylated eNOS (S1177) levels by ≈4- to 5-fold and total Akt and phosphorylated Akt by 4- and 5-fold, it also increased hsp90 association with eNOS by more than 3-fold. Using hydroethidine (HEt), a fluorescent probe for superoxide, we found that hypoxic hearts contained less ethidine (Et) staining than normoxic hearts. Normoxic hearts generated 3 times more superoxide by an N^{ω}-nitro-L-arginine methyl ester (L-NAME)–inhibitable mechanism than hypoxic hearts. Taken together, these data indicate that the association of hsp90 with eNOS is important for increasing ·NO production and limiting eNOS-dependent superoxide anion generation. Such changes in eNOS function appear to play a critical role in protecting the myocardium against ischemic injury. **(*Circ Res*. 2002;91:300-306.)**

Key Words: chronic hypoxia ■ endothelial NOS ■ heat shock protein 90 ■ superoxide anion ■ nitric oxide

Nitric oxide plays an important role in protecting the heart against ischemic injury. *S*-nitrosoglutathione (GSNO), a nitric oxide (·NO) donor, improves functional recovery after ischemia, which is associated with increased cGMP.[1] Chronic hypoxia from birth in a neonatal rabbit model increases recovery of postischemic left ventricular developed pressure (LVDP) compared with recovery in normoxic hearts.[2] It is important to note that nitric oxide synthase (NOS) inhibitors, N^{ω}-nitro-L-arginine methyl ester (L-NAME) and N^{G}-methyl-L-arginine (L-NMA), decrease functional recovery of postischemic LVDP in hypoxic hearts after ischemia but do not decrease recovery in normoxic hearts.[2,3] These findings suggest that chronic hypoxia may alter the function of endothelial nitric oxide synthase (eNOS), the most abundant NOS isozyme in the rabbit heart, to increase cardioprotection.[2]

An increase in the association of heat shock protein 90 (hsp90) with eNOS increases production and activity of ·NO in response to growth factor stimulation.[4] Disruption of this protein-protein interaction decreases ·NO and blocks vasodilation in response to agonists.[4–7] Geldanamycin (GA), which inhibits conformational changes in hsp90[8] and increases oxidative stress by redox cycling,[9] has been shown to decrease ·NO and increase L-NAME–inhibitable superoxide generation in endothelial cells.[6] The role of hsp90 in modulating eNOS function in the heart has not been determined.

In the present study, we examine the role of hsp90 in modulating functional recovery of isolated hearts subjected to global ischemia. Using Western blot analysis, we determined how much hsp90 is associated with eNOS and the extent to which the enzyme is activated based on phosphorylation of eNOS at serine 1177.[10] The levels of superoxide from eNOS in the heart were assessed using NOS inhibitors and hydroethidine (HEt), an oxidant-sensitive fluorescent probe. Al-

Original received January 29, 2002; resubmission received June 7, 2002; revised resubmission received July 19, 2002; accepted July 22, 2002.

From the Division of Pediatric Surgery (Y.S., J.E.B., J.S., K.A.P.) and Cardiothoracic Surgery (J.S.T.), The Cardiovascular Center (Y.S., J.E.B., C.Z., K.A.P.), Free Radical Research Center (K.A.P., J.E.B., Y.S.), and Departments of Biochemistry (J.E.B.), Physiology (C.Z.), and Pharmacology and Toxicology (K.A.P.), Medical College of Wisconsin; and the Section of Cardiothoracic Surgery (J.S.T.), Children's Hospital of Wisconsin, Milwaukee, Wis.

Presented in part at the 74th Scientific Sessions of the American Heart Association, Anaheim, Calif, November 11–14, 2001, and published in abstract form (*Circulation*. 2001;104[suppl II]:II-263).

Correspondence to Yang Shi, PhD, Division of Pediatric Surgery, Medical College of Wisconsin, 8701Watertown Plank Rd, Milwaukee, WI 53226. E-mail yangshi@mcw.edu

Circulation Research is available at http://www.circresaha.org

DOI: 10.1161/01.RES.0000031799.12850.1E

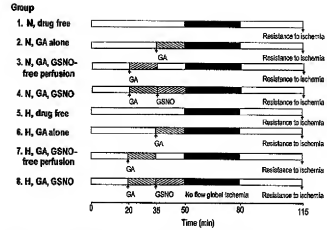

Figure 1. Experimental protocol used to study geldanamycin and GSNO in resistance of normoxic and chronically hypoxic hearts to ischemia. N indicates normoxia; GA, geldanamycin (18 μmol/L); GSNO, S-nitrosoglutathione (10 μmol/L); and H, chronic hypoxia. Open boxes represent aerobic perfusion; hatched boxes, perfusion with drug; and filled boxes, global ischemia.

though ·NO may play an important role in protection, the results of the present study suggest that one of the mechanisms by which hsp90 may protect the heart is by limiting superoxide generation from eNOS.

Materials and Methods

Animals

Animals used in this study received humane care in compliance with the Guide for the Care and Use of Laboratory Animals, by the National Research Council.

Creation of Hypoxia From Birth

Neonatal New Zealand White rabbits were obtained from New Franken Research Rabbits (New Franken, Wis) and were conditioned in normoxic and hypoxic environments as previously described.[2] Details of conditions are presented in an expanded Materials and Methods section, which can be found in the online data supplement available at http://www.circresaha.org.

Perfusion Studies

The protocol for perfusing isolated hearts with GA and subsequent ischemia is described in Figure 1. The protocol for perfusing isolated hearts with HEt and eNOS inhibitors is described in Figure 2. The

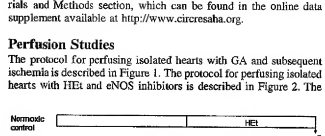

Figure 2. Experimental protocol used to determine the effects of chronic hypoxia on reactive oxygen species generation in the myocardium of hearts. Isolated hearts from normoxic and hypoxic rabbits were perfused with hydroethidine (HEt, 10 μmol/L) and/or L-NAME (400 μmol/L) at the times indicated.

hearts were perfused at 39°C in the Langendorff mode[11] at a perfusion pressure equivalent to 45 mm Hg.[12] The heart and perfusion fluids were immersed in nongassed physiological saline solution within temperature-controlled chambers to maintain the myocardium at 39°C, which is normothermic for rabbit. The standard perfusate was modified Krebs-Henseleit bicarbonate buffer[2]: (in mmol/L) NaCl 118.5, $NaHCO_3$ 25.0; KCl 4.8; $MgSO_4$ 0.6; H_2O 1.2; KH_2PO_4 1.2 (pH 7.4 when gassed with 95% O_2/5% CO_2) in which the calcium content was reduced to 1.8. Glucose (11.1 mmol/L) was added to the perfusate. Before use, all perfusion fluids were filtered through cellulose acetate membranes with pore size 5.0 μm to remove particulate matter.

Assessment of Ventricular Function

Left ventricular function was monitored continuously throughout each experiment as previously described.[13]

Tissue Sample Preparation

Hearts from normoxic and chronically hypoxic neonatal rabbits were isolated and perfused with aerobic bicarbonate buffer for 30 minutes at constant pressure. The free wall of the left ventricle was excised and immediately freeze-clamped between stainless steel tongs precooled with liquid nitrogen. Frozen myocardial tissue samples were powdered in a precooled stainless steel mortar and pestle. The powdered tissue was transferred to a dounce homogenizer with a Teflon pestle and homogenized in modified RIPA buffer (20 mmol/L Tris-HCl, pH7.4, 2.5 mmol/L EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 100 mmol/L NaCl, 10 mmol/L NaF, 1 mmol/L Na_3VO_4, 1 mmol/L Pefabloc, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin A) on ice for 50 strokes. Nuclei and cellular debris were removed by centrifugation (14000g×10 min). The supernatant was transferred to a cold microcentrifuge tube and protein concentrations determined by BCA protein assay (Pierce).

Immunoprecipitation and Western Analysis

Immunoprecipitation and Western analysis protocols were similar to the protocols in a previous report.[2,6] Experimental details for the protocols are provided in the online data supplement.

Detection of Superoxide Anion Generation in Isolated Hearts

The protocol for perfusion of hearts with HEt (10 μmol/L) and eNOS inhibitors, L-NAME (200 and 400 μmol/L), is shown in Figure 2. At the end of the perfusion, hearts were frozen in OCT 4583 and sectioned. Ten micron frozen sections were cut and thaw-mounted on slides. A coverslip was applied to the sections on the slides and images were obtained with a Nikon E600 microscope equipped with epifluorescence (Ex 488 nm, Em 610 nm) and a digital camera. The fluorescent intensity of nuclei in 40 cells from each animal was measured, corrected for background fluorescence in nonnuclear regions using MetaMorph software, and expressed as mean±SD arbitrary units of fluorescence.

Results

Effects of Geldanamycin and GSNO on Functional Recovery

Chronic hypoxia increased postischemic LVDP compared with that obtained in normoxic hearts ($P<0.01$, n=8). Geldanamycin decreased functional recovery of LVDP in normoxic and chronically hypoxic hearts by approximately the same degree ($P<0.01$, n=7 to 9 per group) (Figure 3). GSNO restored functional recovery in GA-treated normoxic and hypoxic hearts treated with GA to levels that were indistinguishable from initial baseline values. To control for the possibility that GSNO-dependent increases in recovery of postischemic LVDP in the GA-treated hearts were due to

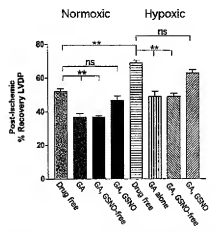

Figure 3. Effects of GA on functional recovery of postischemic LVDP. This bar graph shows LVDP in isolated perfused hearts from normoxic and chronically hypoxic neonatal rabbits. Hearts were perfused with bicarbonate buffer containing buffer alone, buffer containing GA (18 μmol/L), and buffer containing GSNO (10 μmol/L). Protocol pictured in Figure 1 was used to examine the effects of no-flow, global ischemia on functional recovery of LVDP. These data show that GA significantly decreases recovery of postischemic LVDP in both normoxic and hypoxic hearts and that recovery of postischemic LVDP to initial baseline levels can be restored by perfusion with GSNO (**$P<0.01$, n=7 to 9 per experimental test group).

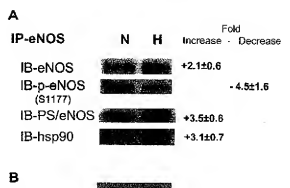

Figure 4. Effects of chronic hypoxia on the activation state of eNOS. A, This composite Western shows that chronic hypoxia in neonatal rabbit hearts increases eNOS protein, decreases phospho-eNOS on eNOS, increases immunodetectable levels of phosphoserine on eNOS and increases association of hsp90 on eNOS compared with eNOS in normoxic hearts. B, Western analysis for hsp90 content in homogenates of normoxic and chronically hypoxic hearts. H indicates hypoxic hearts; N, normoxic hearts; IP, immunoprecipitation; and IB, immunoblot.

perfusion alone, a third group was perfused for the same period of time as the GSNO group with GSNO-free bicarbonate buffer. Perfusion with bicarbonate buffer alone, after perfusion with GA, did not affect recovery of LVDP. The observation that GSNO increased LVDP to baseline levels for both normoxic and hypoxic hearts perfused with GA suggests that regardless of the mechanism by which GA increases susceptibility to ischemia, ·NO from GSNO is sufficient to restore LVDP to baseline values. These data are consistent with the fact that GA shifts the balance of ·NO and superoxide from ·NO toward superoxide anion.[6] These data confirm that shifting the balance of ·NO and superoxide toward superoxide increases susceptibility to ischemic injury and that restoring ·NO increases resistance to ischemia as proposed earlier.[2,14]

Effects of Chronic Hypoxia on the Activation State of eNOS

Previous studies showed that chronic hypoxia increased eNOS activity but not message levels.[2] In the present study, we find by Western analysis that chronic hypoxia increased eNOS levels in heart homogenates by 2.1±0.6-fold ($P<0.05$, n=6) (Figure 4A, first panel). As phosphorylation of eNOS at S1177 indicates the degree of electron flow through eNOS, we next measured phospho-eNOS (S1177) using a site-specific antibody.[6,10,15] The second panel of Figure 4A shows that chronic hypoxia decreased eNOS phosphorylation (S1177) compared with normoxic hearts (−4.5±1.6-fold, $P<0.05$, n=3). At first glance, these data seem to suggest that eNOS in hypoxic hearts might produce less ·NO than eNOS in normoxic hearts, which does not agree with previous findings.[2]

Because hsp90 increases ·NO generation from eNOS[10,15] and decreases superoxide from neuronal NOS (nNOS),[16,17] we next determined the extent to which hsp90 was associated with eNOS in normoxic and chronically hypoxic hearts. Chronic hypoxia increased the association of hsp90 with eNOS compared with normoxic hearts more than 3-fold (3.1±0.7-fold, $P<0.02$, n=6) (Figure 4A, fourth panel). These data demonstrate how important hsp90 is to coupling eNOS activity to L-arginine metabolism for the efficient generation of ·NO.[4,6] Although phospho-eNOS (S1177) may be important for increasing electron flow through the enzyme, increasing the association of hsp90 with eNOS appears to be sufficient to allow chronically hypoxic hearts to generate ≈2 times more ·NO than normoxic hearts.[2] To determine if the increase in association of hsp90 with eNOS is due to a change in hsp90 content, Western analysis of hsp90 in total heart homogenates was performed. Figure 4B shows that chronic hypoxia does not appreciably change the total content of hsp90 in the heart. Taken together, these data support the notion that the association of hsp90 plays an important role in helping eNOS generate ·NO, which protects against ischemic injury.

When the phosphorylation state of eNOS was examined with a general anti-phosphoserine antibody, we found that chronic hypoxia increased immunodetectable levels of phosphoserine on eNOS nearly 3- to 4-fold compared with that found in normoxic hearts ($P<0.05$, n=3; Figure 4A, third panel). As a first step in determining which site(s) on eNOS in rabbits could account for the increase in phosphoserine, we measured by Western analysis phospho-eNOS levels at S116 and T495 that have been reported to mediate eNOS function in other species.[18–20] Unfortunately, the commercially available antibodies did not detect bands of phosphorylation on eNOS from rabbits as they did for eNOS from bovine endothelial cells (Figure 5). The reasons for such differences in detection are unclear at this time but may be because the

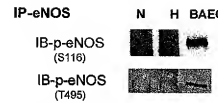

Figure 5. Western analysis for phosphorylation of eNOS. This composite Western of eNOS phosphorylation shows that site-specific antibodies against eNOS at S116 (human) and T495 (human) do not detect bands of phosphorylation on eNOS immunoprecipitated from normoxic and hypoxic rabbit hearts as it does for eNOS immunoprecipitated from cultured bovine aortic endothelial cells. H indicates hypoxic hearts; N, normoxic hearts; and BAEC, bovine aortic endothelial cells.

antibodies were raised in rabbits and/or because the antibodies were against phosphorylation sites in human eNOS, whose amino acid sequence may be different from the sequence for rabbit eNOS.

Effects of Chronic Hypoxia on Akt/Protein Kinase B

Within the signaling cascade for regulation of eNOS, Akt/protein kinase B is located immediately upstream.[15,21,22] On the basis of the data shown in Figure 4A (second panel), we predicted that chronic hypoxia may have altered signaling events leading to decreased phosphorylation of eNOS at S1177. Western analysis of Akt and phospho-Akt in lysates of heart homogenates revealed that chronic hypoxia dramatically decreased total Akt and phospho-Akt in hearts by 4- and 5-fold, respectively (Figure 6A). Because hsp90 did not change with chronic hypoxia, we performed Westerns for hsp90 and phospho-Akt on the same blot to control for loading. Figure 6B confirms findings in Figure 4B that hypoxia has little effect on hsp90 levels and shows that hypoxia seems to specifically decrease phospho-Akt levels, not induce generalized decreases in protein expression. These findings are consistent with the observation that chronic hypoxia decreased phosphorylation of eNOS at S1177. Furthermore, these data suggest that phosphorylation of other residues may regulate eNOS activity. However, using site-

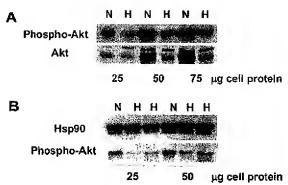

Figure 6. Effects of chronic hypoxia on Akt. A, This Western shows that chronic hypoxia decreases total Akt and phospho-Akt in neonatal rabbit hearts compared with levels in normoxic hearts. B, Western analysis for total hsp90 and phospho-Akt in normoxic and chronic hypoxic hearts. These blots show that chronic hypoxia had no effect on total hsp90 content but dramatically decreased phospho-Akt levels. H indicates hypoxic hearts; N, normoxic hearts.

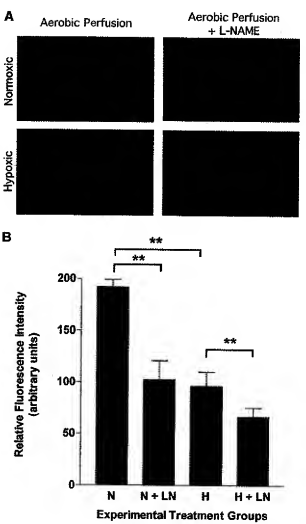

Figure 7. Effects of chronic hypoxia on Et staining in isolated perfused hearts: an index of superoxide anion generation. A, These images show the Et staining in the nuclei of normoxic and hypoxic hearts in the presence and absence of L-NAME. B, This bar graph shows the mean fluorescent intensity of Et staining in the nuclei of the myocardium in normoxic hearts and chronically hypoxic hearts after correction for nonnuclear fluorescence. These data reveal that in normoxic hearts, eNOS generates nearly 3 times more reactive oxygen products that increase Et staining than it does in hypoxic hearts. **$P<0.01$.

specific antibodies against phospho-eNOS (S116) and phospho-eNOS (T495) (human), we were unable to detect similar site-specific phosphorylation of rabbit eNOS, although phosphorylation of bovine eNOS at these sites was clearly evident (Figure 5).

Effects of Chronic Hypoxia on Uncoupled eNOS Activity

Based on the fact that phospho-eNOS (S1177) is a highly conserved site that directly correlates with electron flow through the enzyme,[10] that phosphoserine levels on eNOS have been shown to correlate directly with ·NO generation,[23] and that increased levels of hsp90 association limit superoxide anion generation from NOS,[6,16,17,24–27] we hypothesize that eNOS in chronically hypoxic hearts might be coupled

more efficiently to L-arginine metabolism than eNOS from normoxic hearts. To test this notion, we measured superoxide-dependent conversion of HEt to Et based on a previous report[25] in perfused normoxic and hypoxic hearts. Figure 7A shows that Et staining in normoxic hearts is significantly greater than staining in chronically hypoxic hearts. When the hearts were perfused with L-NAME (which blocks ·NO and superoxide anion production by eNOS[6]) Et staining was reduced to levels seen in hypoxic control hearts. L-NAME also reduced Et staining in chronically hypoxic hearts, albeit to a much smaller extent. When normoxic hearts were perfused with L-NMA, which inhibits ·NO but not superoxide generation from eNOS,[28] Et fluorescence increased markedly (data not shown). These reciprocal differences in the effects of the NOS inhibitors on Et staining in isolated perfused hearts are consistent with the fact that L-NAME blocks superoxide anion from eNOS, whereas L-NMA does not.[28] Image analysis and calculation of relative fluorescent intensities reveals that isolated perfused normoxic hearts generate nearly 3 times more superoxide by an L-NAME–inhibitable mechanism than chronically hypoxic hearts (Figure 7B). A marked increase in eNOS-dependent Et staining in normoxic hearts is consistent with the finding that phospho-eNOS (S1177) is high in normoxic hearts and with the finding that less hsp90 is associated with eNOS in normoxic hearts compared with hypoxic hearts. It is interesting to note that the low levels of L-NAME–inhibitable Et staining in the hypoxic hearts inversely correlated with an increase in general phosphoserine levels on eNOS (Figure 4A, third panel). These findings are consistent with our previous report that chronic hypoxia in neonatal rabbits maximally increases ·NO activity.[2]

Discussion

In this study, we show that geldanamycin (GA) decreases functional recovery of normoxic hearts and inhibits the beneficial effects of chronic hypoxia. Furthermore, we show that the deleterious effects of GA can be reversed by administration of ·NO. As chronic hypoxia increases resistance to ischemia by an L-NAME–inhibitable mechanism,[2,14] our findings suggest that hsp90 and an unidentified phosphoserine site on rabbit eNOS, likely different than S1177, act in concert to increase ·NO production and activity, as suggested in work by others.[4,23] These data suggest that the beneficial effects of chronic hypoxia are more closely related to how much hsp90 associates with eNOS than the magnitude of phosphorylation of eNOS at S1177 alone.[6] The observations that normoxic hearts contain nearly 5 times more phospho-eNOS (S1177) and generate 3 times more eNOS-dependent superoxide, however, are consistent with the fact that phosphorylation of eNOS at S1177 increases electron flow through the enzyme.[10] The relative changes in Et staining in these studies were seen predominantly in the myocytes, consistent with the observations that myocytes representing the majority of heart mass exhibit a diffuse pattern of staining for eNOS that colocalizes with caveolin-3 only at the sarcolemma and t-tubules.[29] On the basis of these observations, we conclude that that hsp90 plays an important role in increasing coupled eNOS activity, which not only increases ·NO production but also preserves ·NO biological activity.[6,16,17,27] Finally, our studies provide new insight into the cellular mechanisms by which adaptation to chronic hypoxia enhances coupled eNOS activity to increase cardioprotection.

Basic science studies using a variety of animal models clearly indicate ·NO plays a central role in cardioprotection. Ischemic preconditioning in rat,[30] canine,[31] and rabbit[32] protects hearts against ischemic reperfusion injury by increasing iNOS. Chronic hypoxia in the rat increases resistance to ischemia in isolated hearts.[33] Chronic hypoxia from birth in rabbits also confers resistance to ischemia.[12,13] Subsequent studies revealed that resistance was due to increased endogenous ·NO production and activity[2,3] and that eNOS, the most abundant transcript for the NOS isozyme family, was unaltered by chronic hypoxia.[3] Such findings indicated that adaptation to chronic hypoxia increases eNOS activity, but not necessarily eNOS mRNA expression to increase resistance to ischemia.[3]

Although the primary purpose of the study was to determine the mechanisms by which chronic hypoxia enhances eNOS activity to increase cardioprotection, a few words about how GA decreases cardioprotection are in order. GA is a well-recognized inhibitor of hsp90.[8] It also contains a semiquinone structure and is thus capable of redox cycling.[9] Accordingly, GA may inhibit functional recovery of isolated hearts by two mechanisms: decreasing ·NO generation via altering hsp90 interactions with eNOS[6] or decreasing ·NO activity via reaction with superoxide.[9] In additional studies, we found that GA decreased nitrite production by isolated hearts by more than half (1.69 ± 0.68 versus 0.77 ± 0.14 nmol/g per mL; $P<0.05$, n=6). In the studies shown in Figure 3, we see that GSNO restores functional recovery of GA-treated hearts to essentially baseline levels. If GA inhibited recovery solely by generating superoxide, then a decrease in nitrite production should not have occurred. If superoxide generated via redox cycling played a major role in decreasing cardioprotection, then GSNO should not have restored recovery of GA-treated hearts to baseline values.

Lucigenin and adriamycin are two well-recognized redox cycling agents that generate superoxide by interacting directly with the reductase domain of eNOS.[34,35] It is important to note that L-NAME does not block superoxide from eNOS when these agents are present.[34,35] The reason is that L-NAME is a substrate analogue inhibitor that only blocks eNOS activity at the arginine oxygenase domain, not the reductase domain.[34,35] With this information in mind, we perfused normoxic and hypoxic hearts with GA and HEt and then analyzed sections for relative levels of Et staining. We found that GA increased Et staining by $45 \pm 5.7\%$ (n=3) in normoxic hearts and $85 \pm 14\%$ (n=3) in hypoxic hearts, which L-NAME blocked as it did earlier.

On the basis that L-NAME is domain specific with respect to inhibiting eNOS-dependent superoxide generation, we conclude that GA increases superoxide anion generation, in a large part, from the arginine oxygenase domain. These findings are consistent with our previous report showing that L-NAME blocked ≈50% of the increase in superoxide generation in A23187-stimulated, GA-treated endothelial cell cultures,[6] reports showing that hsp90 increases eNOS gener-

ation of ·NO,[4,5,36,37] and the report showing that hypoxic hearts contain higher levels of eNOS activity and ·NO biological activity than normoxic hearts.[2] Taken together, these data and reports indicate that although GA can redox cycle to generate superoxide, its ability to inhibit hsp90 plays a major role in the mechanisms by which it decreases cardioprotection in isolated hearts.

To determine how chronic hypoxic increases eNOS activity, we examined the activation state of eNOS. Antibodies against sites of phosphorylation on human eNOS were obtained from commercial sources and used to examine the phosphorylation state of rabbit eNOS. On the basis that chronic hypoxia increases eNOS activity nearly 2-fold,[2] we expected to see a corresponding increase in phospho-eNOS (S1177) levels. Instead, the levels of phospho-eNOS at S1177 were decreased in chronically hypoxic hearts compared with normoxic controls. Further analysis using antibodies to the other phosphorylation sites on rabbit eNOS were unsuccessful, in that clear bands were not detected in samples from rabbits although bands could easily be detected in samples from bovine endothelial cells. The reason for this is unclear at this time. Sequence differences among species or the fact that the antibodies were raised in rabbits are possible explanations. As the antibodies were designed to be site-specific for human sequences, small differences in the amino acid sequence in rabbit eNOS may have been sufficient to prevent detection.

The association of hsp90 with eNOS is a universal mechanism among species for increasing ·NO generation. To date, this protein interaction has been observed in human, rodent, murine, canine, bovine, and ovine endothelial cells and cardiovascular tissues. The importance of this interaction to endothelial biology was recently confirmed by studies showing that hsp90 increased the efficiency of Akt-dependent phosphorylation of eNOS and that specific domains of hsp90 were responsible for delivering and directing Akt to S1179 on bovine eNOS.[38] In light of this information, the lower levels of Akt in homogenates of chronically hypoxic hearts provide a plausible explanation for the low levels of phospho-eNOS (S1177) on eNOS in chronically hypoxic hearts but not the more than 2-fold increase in eNOS activity we reported previously.[2] If one accepts that fact that the association of hsp90 increases eNOS generation of ·NO, then our findings suggest that hsp90 may be more important for increasing eNOS production of ·NO, as well as preserving the biological activity of ·NO, than increasing phospho-eNOS (S1177) levels alone. To determine if the increase in phospho-eNOS (S1177) observed in normoxic hearts still correlated with increased eNOS activity, superoxide, the product of uncoupled eNOS activity was measured. We found that eNOS-dependent Et staining was 3 times greater in normoxic hearts than in hypoxic hearts. Such data also support the idea that phospho-eNOS (S1177) directly correlates with electron flux through eNOS.[10] In the case of the normoxic hearts, however, this increased electron flux was weakly coupled to L-arginine metabolism, resulting in superoxide rather than ·NO generation. In contrast, an increase in general phosphoserine levels on eNOS in hypoxic hearts relative to those in normoxic hearts suggests that other sites of phosphorylation on eNOS also might influence enzyme function and, ultimately, cardioprotection. Future studies aimed at obtaining the full sequence for eNOS will be required to delineate mechanisms by which hsp90 interacts with eNOS in this species.

The possibility that direct protein interactions between hsp90 and eNOS preserves coupled enzyme activity is supported by recent findings by Song et al.[16,17] Using purified recombinant nNOS and hsp90 and spin-trapping with electron spin resonance to quantify ·NO production, Xia and associates[16,17] showed that activation of nNOS in the presence of hsp90 increased ·NO generation. In subsequent studies, they found that hsp90 also inhibited superoxide from nNOS and that this effect was more pronounced at lower L-arginine concentrations than at higher concentrations when hsp90 was present.[27] Another mechanism by which hsp90 might modulate eNOS function is by protecting sites of phosphorylation of eNOS. Using Western analysis, Granger and associates[23] found that VEGF increased phosphoserine residues on eNOS by a protein kinase C (PKC)–dependent mechanism that directly correlated with increased ·NO production and activity. This finding is consistent with those of Ping et al[39] using PKCε-GST-fusion proteins to demonstrate direct interactions between PKCε and eNOS. In the present study, using immunoprecipitation of eNOS and Western analysis, we find that chronic hypoxia markedly increased phosphoserine residues on eNOS even though phospho-eNOS (S1177) decreased. The decrease in phospho-eNOS (S1177) is supported by a marked reduction in total Akt and phospho-Akt, an immediate upstream kinase,[15,21,22] in hypoxic hearts. Our finding that chronic hypoxia increased phosphoserine residues on eNOS is consistent with reports that an increase in phosphoserine increases eNOS activity.[40–42]

These observations reveal how important it is for hsp90 to associate with eNOS when phospho-eNOS (S1177) levels are increased. Failure to increase hsp90 interactions with eNOS results in an inefficient coupling of enzyme activity to L-arginine metabolism and in an increase in eNOS-dependent superoxide generation. Our findings show that chronic hypoxia from birth increases cardioprotection of isolated hearts by increasing the association of hsp90 with eNOS. This critical protein interaction helps to couple eNOS activity to L-arginine metabolism and to limit superoxide anion generation. Such changes in radical species generation by eNOS increase ·NO production and help preserve ·NO activity in the heart, which increases resistance to ischemic reperfusion injury.

Acknowledgments

This work was supported in part by grants AHA 0020453Z (to Y.S.), HL54075 (to J.E.B), and HL61414 (to K.A.P.).

References

1. Konorev E, Tarpey M, Joseph J, Baker J, Kalyanaraman B. *S*-nitrosoglutathione improves functional recovery in the isolated rat heart after cardioplegic ischemic arrest-evidence for a cardioprotective effect of nitric oxide. *J Pharmacol Exp Ther*. 1995;274:200–206.
2. Shi Y, Pritchard KA, Holman P, Raffee P, Griffith OW, Kalyanaraman B, Baker JE. Chronic myocardial hypoxia increases nitric oxide synthase and decreases caveolin-3. *Free Radic Biol Med*. 2000;29:695–703.
3. Baker J, Holman P, Kalyanaraman B, Pritchard K. Adaptation of hearts to chronic hypoxia increases tolerance to subsequent ischemia by increased

nitric oxide production. In: Hudetz A, Bruley D, eds. *Oxygen Transport to Tissue XX*. New York, NY: Plenum Publishing; 1998:203–217.
4. Garcia-Cardena G, Fan R, Shah V, Sorrentino R, Cirino G, Papapetropoulos A, Sessa WC. Dynamic activation of endothelial nitric oxide synthase by Hsp90. *Nature*. 1998;392:821–824.
5. Shah V, Wiest R, Garcia-Cardena G, Cadelina G, Groszmann RJ, Sessa WC. Hsp90 regulation of endothelial nitric oxide synthase contributes to vascular control in portal hypertension. *Am J Physiol*. 1999;277:G463–G468.
6. Pritchard KA Jr, Ackerman AW, Gross ER, Stepp DW, Shi Y, Fontana J, Baker JE, Sessa WC. Heat shock protein 90 mediates the balance of nitric oxide and superoxide anion from endothelial nitric oxide synthase. *J Biol Chem*. 2001;276:17621–17624.
7. Khurana VG, Feterik K, Springett MJ, Eguchi D, Shah V, Katusic ZS. Functional interdependence and colocalization of endothelial nitric oxide synthase and heat shock protein 90 in cerebral arteries. *J Cereb Blood Flow Metab*. 2000;20:1563–1570.
8. Grenert JP, Sullivan WP, Fadden P, Haystead TA, Clark J, Mimnaugh E, Krutzsch H, Ochel HJ, Schulte TW, Sausville E, Neckers LM, Toft DO. The amino-terminal domain of heat shock protein 90 (hsp90) that binds geldanamycin is an ATP/ADP switch domain that regulates hsp90 conformation. *J Biol Chem*. 1997;272:23843–23850.
9. Benchekroun NM, Myers CE, Sinha BK. Free radical formation by ansamycin benzoquinone in human breast tumor cells: implications for cytotoxicity and resistance. *Free Radic Biol Med*. 1994;17:191–200.
10. McCabe TJ, Fulton D, Roman LJ, Sessa WC. Enhanced electron flux and reduced calmodulin dissociation may explain "calcium-independent" eNOS activation by phosphorylation. *J Biol Chem*. 2000;275:6123–6128.
11. Langendorff O. Untersuchungen am uberlebenden Saugertierherzen. *Pflügers Arch Gesamter Physiol*. 1895;61:291–332.
12. Baker R, Boerboom L, Olinger G, Baker J. Tolerance of the developing heart to ischemia: impact of hypoxia from birth. *Am J Physiol Heart Circ*. 1995;268:H1165–H1173.
13. Baker J, Curry B, Olinger G, Gross G. Increased tolerance of the chronically hypoxic immature heart to ischemia: contribution of the KATP channel. *Circulation*. 1997;95:1278–1285.
14. Baker JE, Holman P, Kalyanaraman B, Griffith OW, Pritchard KA, Jr. Adaptation to chronic hypoxia confers tolerance to subsequent myocardial ischemia by increased nitric oxide production. *Ann N Y Acad Sci*. 1999;874:236–253.
15. Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, Sessa WC. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature*. 1999;399:597–601.
16. Song Y, Zweier JL, Xia Y. Heat-shock protein 90 augments neuronal nitric oxide synthase activity by enhancing Ca^{2+}/calmodulin binding. *Biochem J*. 2001;355:357–360.
17. Song Y, Zweier JL, Xia Y. Determination of the enhancing action of HSP90 on neuronal nitric oxide synthase by EPR spectroscopy. *Am J Physiol Cell Physiol*. 2001;281:C1819–C1824.
18. Michell BJ, Griffiths JE, Mitchelhill KI, Rodriguez-Crespo I, Tiganis T, Bozinovski S, de Montellano PR, Kemp BE, Pearson RB. The Akt kinase signals directly to endothelial nitric oxide synthase. *Curr Biol*. 1999;9:845–848.
19. Chen ZP, Mitchelhill KI, Michell BJ, Stapleton D, Rodriguez-Crespo I, Witters LA, Power DA, Ortiz de Montellano PR, Kemp BE. AMP-activated protein kinase phosphorylation of endothelial NO synthase. *FEBS Lett*. 1999;443:285–289.
20. Michell BJ, Chen Z, Tiganis T, Stapleton D, Katsis F, Power DA, Sim AT, Kemp BE. Coordinated control of Endothelial NO synthase phosphorylation by PKC and PKA. *J Biol Chem*. 2001;5:5. Abstract.
21. Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher A. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature*. 1999;399:601–605.
22. Gallis B, Corthals GL, Goodlett DR, Ueba H, Kim F, Presnell SR, Figeys D, Harrison DG, Berk BC, Aebersold R, Corson MA. Identification of flow-dependent endothelial nitric-oxide synthase phosphorylation sites by mass spectrometry and regulation of phosphorylation and nitric oxide production by the phosphatidylinositol 3-kinase inhibitor LY294002. *J Biol Chem*. 1999;274:30101–30108.
23. Wu HM, Yuan Y, Zawieja DC, Tinsley J, Granger HJ. Role of phospholipase C, protein kinase C, and calcium in VEGF-induced venular hyperpermeability. *Am J Physiol*. 1999;276:H535–H542.
24. Pritchard KA Jr, Ackerman AW, Fontana JT, Ou J, Smalley DM, Curtis ML, Stemerman MB, Sessa WC. Native low-density lipoprotein induces endothelial nitric oxide synthase dysfunction: role of heat shock protein 90 and caveolin-1. *Free Radic Biol Med*. 2002;33:52–62.
25. Stepp DW, Ou J, Ackerman AW, Pritchard Jr. KA. Native LDL and minimally oxidized LDL differentially regulate superoxide anion in vascular endothelium in situ. *Am J Physiol*. 2002;283:H750–H759.
26. Deleted in proof.
27. Song Y, Cardounel AJ, Zweier JL, Xia Y. Inhibition of superoxide generation from neuronal nitric oxide synthase by heat shock protein 90: implications in NOS regulation. *Biochemistry*. July 23, 2002; 10.1021/bi026060u. Available at: http://pubs3.acs.org. Accessed August 6, 2002.
28. Vasquez-Vivar J, Kalyanaraman B, Martasek P, Hogg N, Masters BSS, Karoui H, Tordo P, Pritchard Jr. KA. Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proc Natl Acad Sci, U S A*. 1998;95:9220–9225.
29. Hare JM, Lofthouse RA, Juang GJ, Colman L, Ricker KM, Kim B, Senzaki H, Cao S, Tunin RS, Kass DA. Contribution of caveolin protein abundance to augmented nitric oxide signaling in conscious dogs with pacing-induced heart failure. *Circ Res*. 2000;86:1085–1092.
30. Toseki A, Maulik N, Elliott GT, Blasig IE, Engelman RM, Das DK. Preconditioning of rat heart with monophosphoryl lipid A: a role for nitric oxide. *J Pharmacol Exp Ther*. 1998;285:1274–1279.
31. Bolli R, Zughaib M, Li XY, Tang XL, Sun JZ, Triana JF, McCay PB. Recurrent ischemia in the canine heart causes recurrent bursts of free radical production that have a cumulative effect on contractile function: a pathophysiological basis for chronic myocardial "stunning". *J Clin Invest*. 1995;96:1066–1084.
32. Guo Y, Jones W, Xuan X-T, Tang X-L, Bao W, Wu W-J, Han H, Laubach V, Ping P, Yang Z, Qiu Y, Bolli R. The late phase of ischemic preconditioning is abrogated by targeted disruption of the inducible NO synthase gene. *Proc Natl Acad Sci U S A*. 1999;96:11507–11512.
33. Tajima M, Katayose D, Bessho M, Isoyama S. Acute ischaemic preconditioning and chronic hypoxia independently increase myocardial tolerance to ischemia. *Cardiovasc Res*. 1994;28:312–319.
34. Vasquez-Vivar J, Hogg N, Pritchard Jr KA, Martasek P, Kalyanaraman B. Superoxide anion formation from lucigenin: an electron spin resonance spin-trapping study. *FEBS Letters*. 1996;403:127–130.
35. Vasquez-Vivar J, Martasek P, Hogg N, Masters BSS, Pritchard Jr KA, Kalyanaraman B. Endothelial nitric oxide synthase-dependent superoxide generation from adriamycin. *Biochemistry*. 1997;36:11293–11297.
36. Bucci M, Roviezzo F, Cicala C, Sessa WC, Cirino G, Geldanamycin, an inhibitor of heat shock protein 90 (Hsp90) mediated signal transduction has anti-inflammatory effects and interacts with glucocorticoid receptor in vivo. *Br J Pharmacol*. 2000;131:13–16.
37. Gratton JP, Fontana J, O'Connor DS, Garcia-Cardena G, McCabe TJ, Sessa WC. Reconstitution of an endothelial nitric-oxide synthase (eNOS), hsp90, and caveolin-1 complex in vitro: evidence that hsp90 facilitates calmodulin stimulated displacement of eNOS from caveolin-1. *J Biol Chem*. 2000;275:22268–22272.
38. Fontana J, Fulton D, Chen Y, Fairchild TA, McCabe TJ, Fujita N, Tsuruo T, Sessa WC. Domain mapping studies reveal that the M domain of hsp90 serves as a molecular scaffold to regulate Akt-dependent phosphorylation of endothelial nitric oxide synthase and NO release. *Circ Res*. 2002;90:866–873.
39. Ping P, Zhang J, Pierce WM Jr, Bolli R. Functional proteomic analysis of protein kinase Cε signaling complexes in the normal heart and during cardioprotection. *Circ Res*. 2001;88:59–62.
40. Robinson LJ, Busconi L, Michel T. Agonist-modulated palmitoylation of endothelial nitric oxide synthase. *J Biol Chem*. 1995;270:995–998.
41. Garcia-Cardena G, Fan R, Stern DF, Liu J, Sessa WC. Endothelial nitric oxide synthase is regulated by tyrosine phosphorylation and interacts with caveolin-1. *J Biol Chem*. 1996;271:27237–27240.
42. Corson M, James N, Latta S, Nerem R, Berk B, Harrison D. Phosphorylation of endothelial nitric oxide synthase in response to fluid shear stress. *Circ Res*. 1996;79:984–991.